THE DETERMINATION OF VITAMIN D IN MIXED POULTRY FEEDS

by

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INTRODUCTION AND LITERATURE REVIEW

Since shortly after the antirachitic vitamin was first recognized, various workers have been trying to find a good method for determining the potency of various antirachitic substances. Many chemical reagents have been proposed that give color reactions that can be used to assay for vitamin D (71), but they can be used only for relatively pure samples of this vitamin. Feed mixtures, such as those used in this study, contain only small amounts of vitamin D, along with various other sterols which must be removed since they too would give positive reactions. To separate the vitamin D of the mixed feeds from the contaminants by chromatographic or other means is a difficult process. Due to the fact that there are no physico-chemical methods applicable to the assay of complicated mixtures such as poultry feeds, this study has dealt only with the biological method employing chicks as the test animal.

In the U.S.P. assay of vitamin D content of products to be used by humans and other mammals, the line test carried out with albino rats is employed (51, 63, 78, 83). However, since poultry cannot use vitamin D_2 as efficiently as vitamin D_3, whereas mammals do not distinguish between the two forms, rats are not used to assay antirachitic substances for poultry feeding. Therefore a method incorporating chicks as the test animal was developed.

Although other workers had observed that rickets, caused by a deficiency of vitamin D, produced a low bone ash in various

animals, Bethke, Steenbock, and Nelson (6) in 1923 were the first to use bone ash determinations as a diagnosis of a rachitic condition in laboratory animals. Their work was with the rat. In 1925 Hart, Steenbock, and Lepkovsky (42) applied the bone ash method to chickens.

While other workers used the bone ash method only in a qualitative way to determine whether or not a substance had antirachitic properties or acquired such properties under the influence of ultraviolet light, Miller, Dutcher, and Knandel (65) proposed in 1929 that the bone ash method might be useful for determining quantitatively the antirachitic potency of a material. Other workers then started to use this method in a quantitative way; however each used his own process of preparing the bones and determining the ash content. In 1933 St. John, Kempf, and Bond (79) proposed that a standard method be agreed upon so that results from different laboratories would be comparable.

At about the same time, 1932, Griem (31) proposed to the Association of Official Agricultural Chemists that the bone ash method should be studied for possible adoption as an official method for determining the vitamin D content of a feed. He further proposed that any other method that could be applied to the determination of cod liver oil in mixed feeds should be studied also. The rachitic ration first used by Griem (32) was one that had been presented by Hart, Kline, and Keenan in 1931 (43). The method was as follows:

The basal rachitic ration consisted of 59 per cent ground yellow corn, 25 per cent pure flour middlings, 12 per cent casein, 1 per cent calcium carbonate (precipitated), 1 per cent calcium phosphate (precipitated), 1 per cent salt, 1 per cent yeast foam tablet (powder, 5 per cent protein). Groups of six or more one-day old White Leghorn chicks were used, one for negative control purposes, and one or more for each material assayed. The basal ration was supplemented with different levels of the material to be assayed. On the second day the groups were given two fifteen-minute feedings of their respective rations. Beginning the third day the rations were fed ad libitum for thirty-five days at which time the negative control group was rachitic. At the end of the feeding period the birds were killed with ether. One tibia of each bird was removed, cleaned of adhering tissue, numbered, and placed in 95 per cent ethyl alcohol. The bones were crushed, wrapped individually in filter paper, and extracted for seventy-two hours with hot 95 per cent ethyl alcohol. The bones were dried in a moisture oven and the percentage of ash of the moisture- and fat-free bones was determined by igniting in a muffle furnace at 850° C. for one hour. Composite group averages were used for comparative purposes.

In another paper presented to the A.O.A.C. in 1932, Lachat, Halvorson, and Palmer (52) discussed several studies relative to the estimation of vitamin D. One of the things they proposed was a four-week feeding period instead of the five weeks used at that time. With this study in mind, Griem (33) presented the following

revised version of his original method which was adopted as tentative for poultry feed supplements by the A.O.A.C. in 1934 (2).

The basal ration consisted of:

Ground yellow corn	59 per cent
Pure wheat flour middlings	25 " "
Crude domestic acid precipitated casein	12 " "
Calcium carbonate (precipitated)	1 " "
Iodized salt (0.02% KI)	1 " "
Non-irradiated yeast (7% minimum N)	1 " "

The procedure was as follows: Place groups of ten or more one-day-old White Leghorn chicks in screen-bottomed biological cages or battery brooder out of direct sunlight. (Red electric light bulbs are satisfactory as a source of heat for the cages). Reserve one group for negative control purposes, and one or more additional groups for each material to be assayed. Keep distilled water before the chicks at all times. Prepare sufficient basal rachitic ration for the entire feeding period (80 pounds per 100 birds is ample). Prepare the supplemented rations at 8-12 day periods. Supplement the basal rachitic ration with corn oil in a quantity equal to the maximum addition of the oil to be assayed. (This is the ration to be fed to the negative control groups). Supplement the basal ration with different levels of the material to be assayed. Add corn oil to bring the percentage of oil up to that added to the negative control ration. (This is the ration to be fed to the other groups). On the second day give the groups two fifteen-minute feedings of their respective

rations. Beginning the third day feed the rations ad libitum for 28 days. Kill the birds, remove the left tibia of each bird, and clean of adhering tissue. (To facilitate removal of adhering tissue the bones may be placed in boiling water for not over two minutes). Number the bones and place in 95 per cent ethyl alcohol. Crush, wrap individually in filter paper, and extract the bones for twenty hours with hot ethyl alcohol followed by twenty hours with ethyl ether. (Other solvents may be used for this fat extraction). Dry in a moisture oven, and store in a desiccator. Determine the percentage of ash in the moisture- and fat-free bones by igniting in a muffle furnace at approximately 850° for one hour. Compile group ash averages.

Since adoption of Griem's method there has been much work done to modify it. Some of the more important papers reporting on the technic and details of the method and its modifications are discussed below.

There have been a few changes in the rachitic ration used in the method but today it is largely the same as the one first used. One of the changes in the ration removed the calcium carbonate and increased the calcium phosphate from one to two per cent (39). This changed the Ca/P ratio from 1.28 to 1. The yeast content was doubled to increase the riboflavin content. Also 0.2 gram of $MnSO_4 \cdot 4H_2O$ was added to each kilogram of the ration.

The length of assay time has been decreased over the years.

Originally it was believed that five weeks was necessary; however, in 1934 it was shown that the time could be shortened to four weeks (36), and finally in 1937 the period was reduced to three weeks (37). Several workers have attempted to shorten it even more by various methods of depleting the chicks before the start of the assay. Some fed all of the chicks a basal rachitic ration for several days, as is done in the rat line test (17, 19, 83). Since much of the chicks original store of vitamin D is in the yolk, Wei (82) attempted to shorten the feeding period to twelve days by removing the unabsorbed yolk shortly after the chicks were hatched. He reported that the method has no special advantage due to the time and labor involved in the operation. In 1942, De Witt et al. (24) reported that any appreciable decrease in the length of the assay period, under twenty-one days, either with or without a preliminary depletion period, caused a decrease in response between the minimum and maximum bone ash percentages.

For a period it was believed that the bones had to be crushed before they could be extracted completely (53); however it has been shown that this was not necessary (68). It has been shown also that the bones need not be extracted and ashed individually, but that this could be done just as accurately in groups (37, 38, 60). Many different solvents have been studied, but it has been reported that a combination of alcohol and ether is best (59, 66). The method of ashing is usually either overnight at 550° C. or one hour at 850° C.

In an attempt to get away from the difficulty of removing

the flesh from the tibia in a quantitative manner, the toe ash method has been used by some workers (4, 8, 28). The middle toe of either foot is removed at the middle joint. This is a more rapid procedure than removing the tibia and is better since the chicks need not be killed. The toe is ashed either green[1] or after it is extracted and dried in the same manner as the tibia. Of course, the green toe ash procedure causes a decrease in accuracy due to the smaller range between the minimum and maximum ash content; however, the shortened procedure possibly compensates for the smaller difference. The results obtained on the extracted toe agree favorably with those found on the tibia; however there again is a smaller range between the minimum and maximum bone ash (9, 12, 13, 26). The toe ash method is not official but Friedman (28) has proposed that it be studied collaboratively as an alternative method.

Wei (82) has recently proposed using either the lower or upper beak ash in place of the tibia ash as a procedure for the determination of vitamin D. This method gives a range between minimum and maximum ash content approximately as large as that of the tibia ash method. He reported that this criterion of calcification shows promise as another method to determine the vitamin D content of feeds.

According to Johnson (47, 48) the removal of the epiphyseal

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[1]In the green toe ash method the toe is ashed without being extracted or dried. It is weighed and ashed just as it is removed from the chick.

cartilages from the tibia of the chick facilitated rapid and complete removal of adhering tissue from the bone. The resulting bone analyzes about 8.5 per cent greater ash than does the opposite tibia with the cartilages left intact. The variation between the ash values within groups is slightly less for bones with the cartilages removed than for those with cartilages intact.

Other workers (64) have reported on the use of the femur ash method. However, this has no advantages over the tibia and has the disadvantage that the femur is more difficult to remove than is the tibia.

The use of the X-ray in measuring the tarso-metatarsal distance has been reported to be a good method for the determination of vitamin D (5, 49), but too few laboratories have access to an X-ray machine for the method to be adopted on a wide scale.

In 1951 the tibia ash method for the determination of vitamin D in poultry feed supplements was made official by the Association of Official Agricultural Chemists. Since this is the method that was used throughout the trials reported herein, it has been included in the section on methods.

The purpose of the study reported herein was to determine whether a modification of the official method for the determination of vitamin D in poultry feed supplements could be used for the determination of vitamin D in mixed feeds.

In 1935 the quantitative biological detection of vitamin D

in mixed feeds, as measured by antirachitic potency, was studied by Griem (34). Two lines of approach to the solution of the problem were considered: first, the extraction of the proprietary feed with a crude fat solvent, followed by subsequent vitamin D assay of the extract, and second, dilution of the mixed feed with the rachitic ration used for the vitamin D assay, followed by a feeding trial to determine degrees of calcification produced. Griem reported that the second method seemed promising but that more work was needed. However, a library search has disclosed no more work along this line. Griem's first method was not studied further due to the fact that it was found that the extraction of vitamin D was not quantitative. This present study, then, is a continuation of the work started by Griem on his second method.

EXPERIMENTAL PROCEDURES

Method

The official method for the determination of vitamin D in poultry feed supplements is as follows (1):

(Applicable to fish and fish-liver oils and their extracts and to materials used for supplementing vitamin D content of feeds. Not applicable to irradiated ergosterol products nor to irradiated yeast unless recommended for poultry. This assay is comparison, under conditions specified, of efficiency of product

under assay with that of U.S.P. Vitamin D Reference Standard in controlling ash content of bones of growing chicks.)

Basal Rachitic Ration. The basal ration is a uniform mixture in proportions designated of following ingredients, which have been finely ground:

Yellow corn, ground	58 per cent
Wheat flour middlings or gray wheat shorts	25
Casein, crude, domestic, acid precipitated	12
Calcium phosphate, precipitated	2
Salt, iodized (0.02% KI)	1
Yeast, non-irradiated (7% minimum N)	2

To each kg of the above mixture add 0.2 g of $MnSO_4 \cdot 4H_2O$.

Determination. Provide cages with screen bottoms and keep chicks away from sunshine or other sources of actinic light that may influence calcification. Keep cages in room in which wide variations in temperature are prevented (constant temperature preferred). Unless temperature of the room is adequately controlled, provide each cage with suitable electric heating device. Start all birds to be used in one assay on the same day and keep all conditions of environment uniform for all groups in assay.

Make assay on groups of one or two day old White Leghorn chicks as specified below. Provide for one or more negative control groups that receive no vitamin D, three or more positive control groups that receive graduated levels of vitamin D from U.S.P. Vitamin D Reference Standard and one or more assay groups

for each product to be assayed. Have positive control groups and assay groups consist of not less than 20 birds each and negative control group consist of not less than 10 birds. Make up rations for all groups in assay from one batch of basal ration. Add the reference standard oil to basal ration in such quantities as to produce measurable increase in the per cent bone ash above that obtained in negative control group (it is not possible to make comparison if maximum bone ash is obtained). Add assay product to basal ration in such quantities as to permit direct comparison in response of assay and positive control groups. To the basal ration of negative control group add corn oil equal in quantity to maximum quantity of oil fed to any group in the assay, and add corn oil to rations of the other groups until the quantity of corn oil and oil containing vitamin D is equal to quantity of corn oil added to the ration of the negative control group. Feed chicks in respective groups prescribed ration and water (natural or distilled) ad libitum 21 days. Discard all chicks that show abnormality or disease not related to vitamin D deficiency. At least 15 chicks must remain in each reference or assay group used in calculating vitamin D potency of assay product.

Kill chicks; remove left tibia of each bird and clean of adhering tissue. (To facilitate removal of adhering tissue bones may be placed in boiling water for not more than two minutes. Bones may be preserved in alcohol for extraction). Completely extract bones with suitable solvent or solvents. (20 hours with hot alcohol followed by 20 hours with ether, and bones may be

crushed to facilitate extraction). Dry extracted bones to constant weight in moisture oven, cool in desiccator, and weigh. Ash moisture- and fat-free bones from each group in muffle furnace to constant weight at any given temperature between 450° and 550°, or if preferable one hour at ca 850°. (Ash determination may be made on individual bones if desired). Cool ash in a desiccator and weigh. Use consistently throughout any one assay the specific procedure adopted for extracting, drying and ashing the bones.

Interpretation of Results. One International Chick unit of vitamin D is equal in biological activity for chicks to one unit of vitamin D in U.S.P. Vitamin D Reference Standard in this method of assay. Product under assay meets its declared vitamin potency in International Chick units of vitamin D if percentage of ash in moisture- and fat-free bone produced in assay group by given number of units of vitamin D is equal to or greater than percent ash produced by same number of units of vitamin D from U.S.P. Reference Standard.

Techniques Used in the Present Studies

The yellow corn used in the preparation of the ration was ground in a hammermill through the finest screen in order to make a homogeneous mash. The other ingredients were already finely ground, and nothing further was done to prepare them. The ingredients were thoroughly mixed in a Gilson mechanical feed

mixer[1]. A premix of the vitamin D was made by adding the oil to two or three pounds of the feed and thoroughly mixing by hand. The premix was then added to the remaining feed and blended in the mixer. Enough feed was prepared per batch to last the full three-week feeding period. During this period each chick ate on the average a little less than one pound of feed; therefore one hundred pounds of the total ration was mixed for each hundred chicks.

The amount of U.S.P. Vitamin D Reference Standard that was required for each separate ration was weighed on the analytical balance and diluted to the same volume (one milliliter for each pound of feed) with cottonseed oil (Wesson Oil) instead of corn oil as specified in the method[2]. Without dilution it would have been impossible to mix the small amount of oil throughout the entire ration. Furthermore each ration contained the same amount of oil. Oil of the same amount was added to the feed for the negative control chicks.

As soon as the chicks were received they were wing banded and given an intranasal vaccination for Newcastle Disease[3]. They were weighed, placed in their respective pens and fed immediately. The chicks were all raised in Bussey chick starting

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[1]Gilson Brothers Manufacturing Co., Fredonia, Wisconsin.

[2]Cottonseed oil was used since it is a well known fact that it does not become rancid as quickly as corn oil, and the new U.S.P. XIV Vitamin D Standard is a solution of D_3 in cotton seed oil.

[3]Lederle intranasal Newcastle Disease vaccine was used.

batteries which were kept out of direct sunlight as much as possible. The chicks were protected from the ultraviolet light of the sun by keeping them behind window glass. For twenty-one days the chicks were fed their respective rations ad libitum and tap water was kept before them at all times. The chicks were weighed weekly and, except during the first week, any chicks that were found to be in the wrong pen were discarded. Several times in these trials a number of the chicks were found to have gotten into the wrong pen, but since they were only one week old and not yet eating much feed and since they could have been mixed up only a few days, it was felt that they should be returned to their proper pen and continued on the trial. At the end of the twenty-one-day feeding period all of the chicks remaining in the trial were put into containers (each group separately) and killed with chloroform.

In the first trial, six criteria of calcification were used to determine the vitamin D potency of the feed. These were the ash contents of the tibia, as is used in the official assay; the left toe, moisture- and fat-free; the right toe, on both the green and dry basis; the upper beak; and the lower beak. In the remaining trials the right toe was ashed on the green basis only. This provided five criteria of calcification.

The tibia was removed by cutting through the femur with a pair of heavy scissors. After the skin was pulled down to the foot, the tibia was freed by cutting through the joint between the tibia and the foot with a scalpel and scissors. In order

to facilitate the removal of the flesh from the bone it was placed in boiling water for one minute. The flesh and fibula were picked off and any fragments were removed by rubbing the bone with a dry cloth. The cartilage was not removed from the ends of the bones. Each group of bones was wrapped in separate filter paper and numbered. The bones were stored in 95 per cent alcohol until they could be extracted. The extraction was carried out in a Soxhlet fat extractor using 95 per cent alcohol for 20 hours followed by ether for 20 hours. The bones were dried in the air, dried overnight in a moisture oven, and then ashed in a muffle furnace for one hour at 850° C.

The toes were removed by cutting through the middle joint with a pair of scissors. The right toe was weighed immediately and the ash determined on the green basis. The toes were dried overnight before they were ashed, but only in the first trial were they weighed after they were dried. The left toe was extracted and dried in the same way as the tibia, except the flesh was not removed from the toes.

The hard part of the upper and lower beaks was removed with a pair of scissors. The lower beak was cut at the base of the tongue and the flesh "V" in the center was removed. The upper beak was cut off at the base of the nostrils so that only the hard part was removed. The fleshy part was removed from the inside of the upper beak. The beaks were treated in the same manner as the tibia and the left toe.

In order to be sure of the vitamin D potency of the feeds to

be assayed in Trials I, II, and III, the vitamin D was added in the form of the U.S.P. Reference Standard which contains 400 International Chick units of crystalline vitamin D_3 per gram of solution in cottonseed oil. Therefore since the potency of the test feeds was definitely known, the accuracy of the assays could be determined. In Trial IV the vitamin D was supplied as Delsterol.

Outline of the Trials Conducted

Trial I. In this trial 113 White Leghorn chicks[1] were divided into seven groups, A through G, as shown in Table I.

The test feed in this trial was a commercial broiler mash[2] that was removed from the manufacturer's mixer before the vitamin premix was added. Therefore this feed should have contained little if any vitamin D.

Trial II. In this trial 169 chicks[3] were divided into ten groups, H through Q, as is shown in Table 2. When the chicks within each group were killed they were subdivided into random subgroups and the bones of the subgroups were analyzed separately. Thus for each group there were three ash percentages determined - the two subgroups and the composite. This was done in order to

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[1]The chicks were straight run Single Comb White Leghorns from the Baker Hatchery, Abilene, Kansas.

[2]The broiler mash was furnished by the Mid West Mills Co., Inc., Abilene, Kansas.

[3]The chicks were Single Comb White Leghorn Cockerels purchased from Baker's Hatchery, Abilene, Kansas.

get a better idea of the precision of the method and of differences that might have significance.

The test feed for this and the third trial was composed of the following ingredients plus certain minerals[1]:

Ground white corn	60.5 lbs.
Wheat bran	4
Soybean meal	27
Dried skim milk	2
Steamed bone meal	1
Non-irradiated yeast	3
Iodized salt	0.5
Calcium carbonate	2
Riboflavin	5 grams
Choline chloride	9
Calcium pantothenate	1
Niacin	5
Animal protein factor	23
$MnSO_4$	0.25

Feed X is the feed just as shown above; feed Y is the same except for the addition of one per cent more calcium carbonate in order to increase the Ca/P ratio.

Trial III. In this trial 185 chicks[2] were divided into ten groups, A' through J', as is shown in Table 3. The feed X used

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[1] This feed is a modification of the K.S.C. High Efficiency Chick Starter.

[2] The chicks were straight run Single Comb White Leghorns hatched at the K.S.C. poultry farm.

in Trial III was the same as the feed X used in Trial II. However, in this trial it was used at the ten per cent level since this is more nearly the level that would be required in the assay of mixed feeds by the method used in the present study. Feed Z is the same as feed X, except for the addition of 3.39 per cent of K_2HPO_4. This was calculated to give a Ca/P ratio of 1.0, which is the same as that of the basal ration.

Due to a shortage of U.S.P. Vitamin D Reference Standard the vitamin D added to ration D', the positive control, was in the form of Delsterol.[1]

In this trial the chicks were killed on the twenty-first day instead of the twenty-second as in the other trials.

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[1] A vitamin D supplement manufactured by E.I. Du Pont de Nemuors and Co., Inc. containing 1500 I.C. units of vitamin D per gram.

Table 1. Rations fed to the chicks of Trial I.

Group	Number of chicks[a]	I.C. units of vitamin D per 100g of feed	Per cent of test feed	Per cent of basal ration	Ca/P ratio of final ration[b]
A	12	0	0	100	0.976
B	10	25	0	100	0.976
C	20	7	0	100	0.976
D	19	12	0	100	0.976
E	19	7	25	75	1.057
F	15	12	25	75	1.057
G	18	0	25	75	1.057

[a]The number of chicks that remained at the end of the assay period. The variations were due to dead and discarded chicks, since all groups originally contained 20 chicks except A and B which contained 12 and 11 chicks respectively.

[b]The calcium and phosphorus contents of the feeds were determined by the A.O.A.C. method.

Table 2. Rations fed to the chicks of Trial II.

Group	Number of chicks[a]	I.C. units of vitamin D per 100g of feed	Per cent of feed X	Per cent of feed Y	Per cent of basal ration	Ca/P ratio of final ration
H	13	0	0	0	100	1.075
I	20	7	0	0	100	1.075
J	19	12	0	0	100	1.075
K	13	25	0	0	100	1.075
L	15	0	25	0	75	1.301
M	19	7	25	0	75	1.301
N	18	12	25	0	75	1.301
O	15	0	0	25	75	1.449
P	18	7	0	25	75	1.449
Q	19	12	0	25	75	1.449

[a]The number of chicks that remained at the end of the assay period. All groups started with 20 chicks except H, L, and O which originally contained 13, 16, and 15 respectively.

Table 3. Rations fed to the chicks of Trial III.

Group	Number of chicks[a]	I.C. units of vitamin D per 100g of feed	Per cent of feed Z	Per cent of feed X	Per cent of basal ration	Ca/P ratio of final ration
A'	13	0	0	0	100	0.964
B'	27	7	0	0	100	0.964
C'	27	12	0	0	100	0.964
D'	14	25	0	0	100	0.964
E'	12	0	25	0	75	0.978
F'	20	7	25	0	75	0.978
G'	20	12	25	0	75	0.978
H'	13	0	0	10	90	1.06
I'	19	7	0	10	90	1.06
J'	20	12	0	10	90	1.06

[a] The number of chicks that remained at the end of the assay period.

Trial IV. In this trial 165 chicks[1] were randomized into eleven groups, K' through U', as presented in Table 4.

Feed V is the same as the broiler mash that was used in Trial I, except that Delsterol was added to give it a vitamin D potency of 400 I.C. units per pound.[2] Feed W is the K.S.C. High Efficiency Chick Starter, which according to the formula contains 600 I.C. units of vitamin D per pound.[3]

This trial was set up exactly as in the actual assay of a mixed feed for vitamin D. The test feeds were added in such amounts that the potency desired in the final mixture with the basal rachitic ration would be obtained.

The size of the groups in this trial was small due to a high death rate during the first week that was caused by an infection which apparently came from the hatchery. Group K' originally consisted of 15 chicks while the remaining groups contained 20 chicks each.

The replicated groups in this trial were raised completely separate from each other. Their rations were mixed separately and stored in individual cans.

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[1]The chicks were Single Comb White Leghorn cockerels which were hatched at the K.S.C. poultry farm.

[2]The broiler mash (V) was furnished by the Mid West Mills Co. Inc., Abilene, Kansas, and was the same as used in Trial I.

[3]The K.S.C. High Efficiency Chick Starter was furnished by the K.S.C. Poultry Department.

Table 4. Rations fed to the chicks of Trial IV.

Group	Number of chicks[a]	I.C. units of vitamin D per 100g of feed	Per cent of feed W	Per cent of feed V	Per cent of basal ration	Ca/P ratio of final ration
K'	11	0	0	0	100	0.936
L'	13	7	0	0	100	0.936
M'	14	7	0	0	100	0.936
N'	18	12	0	0	100	0.936
O'	17	12	0	0	100	0.936
P'	13	7	5.32	0	94.68	0.979
Q'	16	12	9.12	0	90.88	1.007
R'	15	12	9.12	0	90.88	1.007
S'	15	7	0	7.95	92.05	0.964
T'	16	12	0	13.64	86.36	0.986
U'	17	12	0	13.64	86.36	0.986

[a] The number of chicks that remained at the end of the assay period. Group K' originally consisted of 15 chicks while the remaining groups contained 20 chicks each.

RESULTS

Trial I

The ash contents of the tibiae of the chicks of Trial I varied from 32.1 to 45.4 per cent, a range of 13.3 per cent (Table 6). Except in the case of the groups of chicks receiving no vitamin D, there was satisfactory correlation between the ash contents of the tibiae of chicks on the basal rachitic ration and those on the test ration composed of 25 per cent broiler feed plus 75 per cent basal rachitic ration. The fact that the lines of the graph (Fig. 1) cross cannot be explained, but the differences between the ash contents are less than one per cent ash and are within the margin of experimental error which is inherent in the method (80).

The results obtained using the extracted toe ash method with the left toe compare favorably with those of the tibia ash method (Table 6). The ash content varied from 10.6 to 18.0 per cent, a range of 7.4 per cent. This is considerably less than the range obtained using the tibia ash method; however as judged by the correlation of toe ash and levels of vitamin D fed, this method was as suitable as the tibia ash for assay purposes. In contrast to the results obtained when using the tibia ash method, there was no cross over of the graphs representing the calcification of the extracted toe produced by the two rations at 0, 7, and 12 I.C. unit levels (Fig. 2).

The green toe ash method yielded results which were not as good as those of the extracted toe method (Table 6, Fig. 3). The range of ash content in this case was only from 2.9 to 5.7 per cent or a maximum difference of only 2.8 per cent. The method employing the right toe on a dry basis was not used after this trial, since it failed to show the accuracy that would be needed in the practical application of the method for the determination of vitamin D in mixed feeds.

For some unexplained reason the agreement in the data on the ash contents of both the lower and upper beaks obtained using the two different rations was better at the 12 I.C. unit level than at the lower levels (Figs. 4 and 5). The ranges between the minimum and maximum ash contents of both beaks were practically identical (14 per cent); however the ash contents of the lower beaks was consistently about 9 per cent higher than those of the upper beaks at identical levels of vitamin D in feed the chicks received.

Except in the data on the green toe, the ash contents of the bones of the chicks fed the basal rachitic ration plus 25 per cent of the broiler mash were greater than those of the chicks fed the basal ration only when they were getting no vitamin D. This might possibly be due to an influence of the higher Ca/P ratio of the broiler ration on the bone calcification when vitamin D was not present. This action, however, apparently was overshadowed by the influence of the vitamin D in the rations fed to the other groups of chicks of this trial.

Table 6. Ash content of the tissues (Trial I).

Ration[a]	I.C. units of vitamin D per 100g of feed	Tissues ashed: Tibia	Left toe	Right toe (dry)	Right toe (green)	Upper beak	Lower beak
		Per cent bone ash					
Basal only	0	32.1	10.6	9.0	3.6	13.5	22.1
	7	39.6	14.8	9.0	3.8	19.4	27.9
	12	42.1	17.2	11.6	4.5	24.2	33.3
	25	45.4	18.0	13.7	5.7	27.5	36.0
Basal + 25% broiler	0	34.7	12.2	7.6	2.9	16.6	25.6
	7	38.9	15.1	9.9	4.0	21.7	30.2
	12	43.0	18.0	11.2	4.8	24.6	32.6

[a] See text for the description of the feeds.

Trial II

In this trial the range between the minimum and maximum ash contents of the tibiae was only 10.6 (Table 7, Fig. 1). This is 2.7 per cent less than in Trial I.

The calcification produced by feed Y added to the basal ration was greater than that produced by either the basal rachitic ration alone or when feed X was added to the basal ration. This possibly was due to the fact that the Ca/P ratio of feed Y was 2.57 (a ratio of 1.45 in the mixture of feed Y plus the basal ration), while that of feed X was only 1.98 (a ratio of 1.30 in the mixture fed) as compared to that of the basal ration, 1.075. The accuracy of the determinations, as judged by the correlation between the ash contents of the bones of the chicks fed the test mixtures and that of the chicks fed the basal ration only, when using feed X was not as great as would be desired, but the error probably would not be excessive.

The differences in the calcification produced by the mixture of 75 per cent basal ration plus 25 per cent feed Y and the basal ration alone were not as great when using the other four criteria as it was when measured by the tibia ash method (Table 7, Figs. 2, 3, 4, and 5). In fact, when using the extracted toe ash method the accuracy was better when feeding feed Y than when feeding feed X. The range of ash content between minimum and maximum calcification in the extracted toe was 5.3 per cent which was 2.1 per cent less than that found in Trial I.

Table 7. Percentage of bone ash in Trial II.

Tissue ashed	I.C. units of vitamin D per 100g of feed	Type of ration fed: Basal ration only — Subgroups I	Basal ration only — Subgroups II	Basal ration only — Composite	Basal + 25% X a — Subgroups I	Basal + 25% X a — Subgroups II	Basal + 25% X a — Composite	Basal + 25% Y a — Subgroups I	Basal + 25% Y a — Subgroups II	Basal + 25% Y a — Composite
		Per cent bone ash								
Tibia	0	31.2	31.4	31.3	31.5	31.0	31.3	32.5	31.4	31.9
	7	35.3	35.1	35.2	37.7	35.4	36.6	39.1	37.7	38.3
	12	38.4	39.8	39.0	37.6	37.9	37.8	40.9	40.3	40.6
	25	41.2	42.5	41.9						
Left toe	0	11.6	11.7	11.7	11.1	11.1	11.1	11.7	11.2	11.4
	7	14.3	13.7	14.0	14.3	13.2	13.8	13.9	13.9	13.9
	12	16.5	16.2	16.4	15.3	14.7	15.0	17.0	15.3	16.2
	25	16.6	17.3	17.0						
Right toe (green)	0	3.6	3.5	3.5	3.8	3.6	3.7	3.9	3.7	3.8
	7	4.4	4.3	4.3	4.8	4.5	4.6	4.7	4.9	4.8
	12	5.1	5.8	5.3	5.0	5.1	5.0	5.4	5.1	5.2
	25	6.1	5.9	6.0						
Upper beak	0	17.6	15.2	16.4	17.7	15.7	16.8	17.5	16.2	16.8
	7	20.6	21.5	21.0	23.1	21.5	22.4	22.1	22.2	22.2
	12	24.8	26.4	25.5	24.8	24.2	24.5	26.3	24.5	25.5
	25	27.9	26.8	27.4						
Lower beak	0	23.9	24.0	23.9	25.6	24.3	25.0	24.6	23.3	23.9
	7	28.2	29.9	29.1	29.5	29.0	29.3	30.0	30.0	30.0
	12	31.8	34.1	32.8	31.3	31.9	31.6	33.3	32.7	33.0
	25	35.3	34.7	34.9						

[a] See text for description of feeds.

As in Trial I, the green toe ash method did not give as satisfactory results as those of the extracted toe ash method. However on the average the differences were not excessive.

There was little difference in the results obtained by the use of the upper and lower beak ash methods. The range between the minimum and maximum ash content was 11.0 per cent for both the upper and lower beaks. Both gave results that could be interpreted satisfactorily in the determination of vitamin D. Again the basal ration plus feed Y produced the greatest calcification.

Trial III

The range between the minimum and maximum ash contents of the tibiae from chicks of this trial was 12.0 (Table 8, Fig. 1). This was larger than that of Trial II, but not as large as that of Trial I.

The difference in ash contents of the tibiae of chicks fed the basal ration supplemented with feed Z and the tibiae of the chicks fed the basal ration alone was less when the feed contained 12 I.C. units of vitamin D per 100 g of feed than at the other levels used. Even though feed Z had a Ca/P ratio practically the same as that of the basal rachitic ration (0.978 and 0.964, respectively), it produced greater calcification than did the basal ration alone. This might have been caused by the fact that, even though the Ca/P ratio was low, feed Z had a one per cent higher total calcium and phosphorus content than did the basal ration. The accuracy of the determinations at the 12 I.C. units level was

much poorer in the case of the ration supplemented with feed X than it was at the 7 I.C. units level.

Except when feed Z, containing 12 I.C. units of vitamin D per 100 g of feed was used, the accuracy of the determinations of the extracted toe ash method was good (Table 8, Fig. 2). Also in the case of the green toe ash method the accuracy was good at most levels of vitamin D supplementation (Fig. 3).

The best accuracy achieved in these studies was obtained when the basal ration was supplemented with feed X and the lower beak ash method was used (Fig. 4).

Trial IV

Trial IV was set up as in the actual assay of a mixed feed. The mixed feeds used were a commercial broiler mash which contained Delsterol at the rate of 400 I.C. units of vitamin D per 100 g of feed (feed V) and a starting mash which contained 600 I.C. units of vitamin D per 100 g (feed W).

The results of the analysis of the vitamin D contents of each feed by ashing the five different bones agree satisfactorily. In each case, except the green toe ash method, when feeds V and W were added to the basal ration so that the final mixture was calculated to contain 7 I.C. units per 100 g of ration, the ash content of the bones obtained in the assay of feed V were greater than in the assay of feed W (Figs. 6, 7, 8, 9, 10, Table 9).

Table 8. Ash content of the tissues (Trial III).

Tissue	I.C. units of vitamin D per 100g of feed	Type of ration fed: Basal ration only — Subgroups I	Basal ration only — Subgroups II	Basal ration only — Composite	Basal + 25% [a] — Subgroups I	Basal + 25% [a] — Subgroups II	Basal + 25% [a] — Composite	Basal + 10% X [a] — Subgroups I	Basal + 10% X [a] — Subgroups II	Basal + 10% X [a] — Composite
		Per cent bone ash								
Tibia	0	29.6	31.1	30.3	32.6	31.7	32.1	31.7	30.9	31.3
	7	36.9	37.5	37.2	38.3	39.5	39.0	38.6	37.8	38.1
	12	41.6	40.4	41.0	40.3	40.9	40.6	44.3	42.3	43.3
	25	43.4	41.4	42.3						
Left toe	0	10.3	11.6	10.9	11.2	10.5	10.8	11.1	10.8	10.9
	7	14.8	14.7	14.8	14.7	14.9	14.8	14.9	14.1	14.4
	12	17.7	16.7	17.2	15.7	15.7	15.7	16.2	17.2	16.7
	25	18.2	17.2	17.7						
Right toe (green)	0	3.9	4.6	4.2	4.3	3.8	4.1	4.0	3.6	3.8
	7	5.1	4.9	5.0	5.2	4.9	5.0	5.2	4.7	4.9
	12	6.0	6.0	6.0	5.6	5.6	5.6	5.7	5.8	5.8
	25	6.6	6.2	6.4						
Upper beak	0	12.5	16.3	14.2	16.5	14.3	15.4	13.5	12.9	13.1
	7	19.9	21.1	20.5	23.5	23.2	23.3	23.2	20.8	21.8
	12	25.5	25.5	25.5	26.5	26.2	26.4	26.8	26.4	26.6
	25	27.4	28.0	27.7						
Lower beak	0	20.7	22.4	21.5	24.4	23.1	23.7	21.1	21.3	21.2
	7	29.0	29.5	29.3	31.3	31.4	31.3	30.9	28.4	29.6
	12	34.0	34.2	34.1	33.8	34.1	34.0	34.1	34.0	34.1
	25	36.3	35.4	35.9						

[a] See text for the description of the feeds.

Table 9. Ash content of the tissues (Trial IV).

Tissue ashed	I.C. units of vitamin D per 100g of feed	Type of ration fed: Basal only	Basal + V[a]	Basal + W[a]
		Per cent bone ash		
Tibia	0	33.6		
	7	37.9	38.9	37.7
	7	36.6		
	12	42.2	43.7	42.6
	12	41.4	41.5	43.7
Left toe	0	10.9		
	7	14.1	14.2	14.1
	7	13.9		
	12	16.0	16.8	15.6
	12	16.0	16.4	16.3
Right toe (green)	0	3.53		
	7	4.61	4.34	4.36
	7	4.40		
	12	5.32	5.27	5.10
	12	5.14	5.01	5.21
Upper beak	0	15.5		
	7	20.8	21.2	20.7
	7	20.2		
	12	24.5	25.5	25.0
	12	24.9	24.0	25.1
Lower beak	0	23.1		
	7	28.3	27.9	27.1
	7	26.8		
	12	31.2	32.0	31.4
	12	30.3	30.9	31.5

[a]See text for a description of the feeds.

The assay of feed W showed that it contained approximately the amount of vitamin D that had been stated in the formula, while feed V possibly contained slightly more than the stated potency.

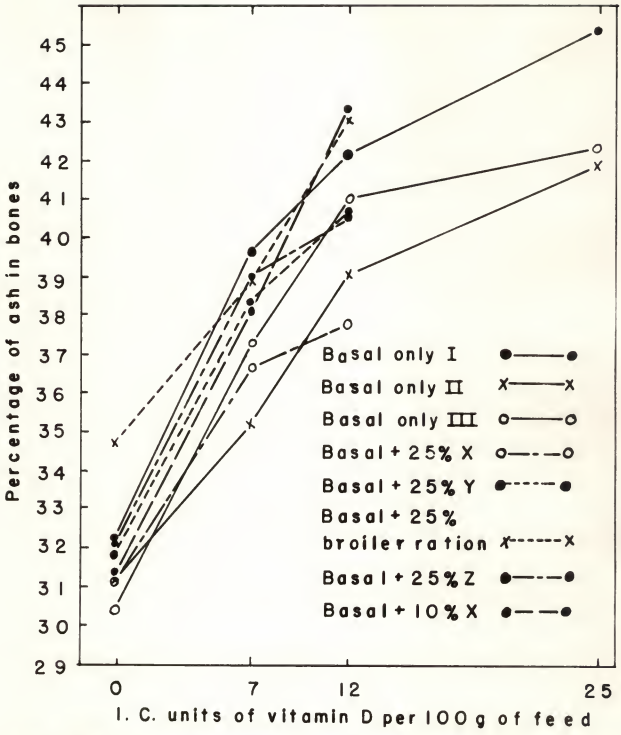

Fig. 1. Percentage of ash in the tibia of Trials I, II, and III.

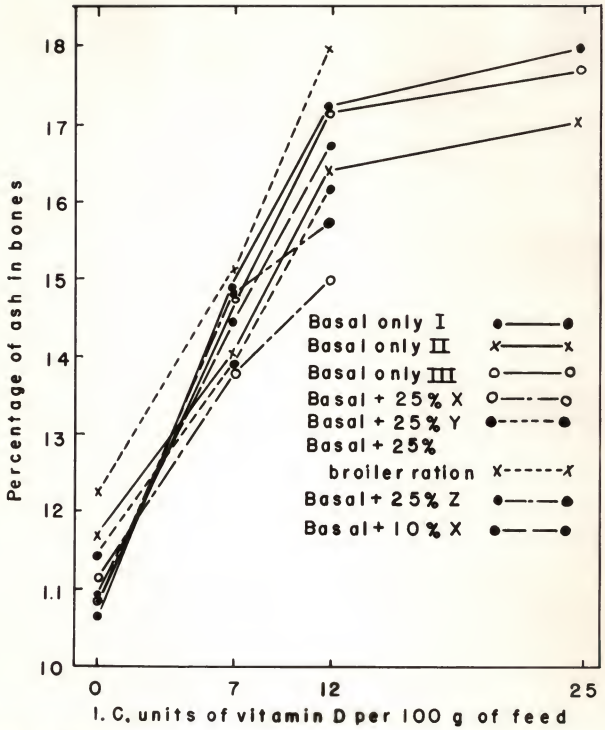

Fig. 2. Percentage of ash in the left toe of Trials I, II, and III.

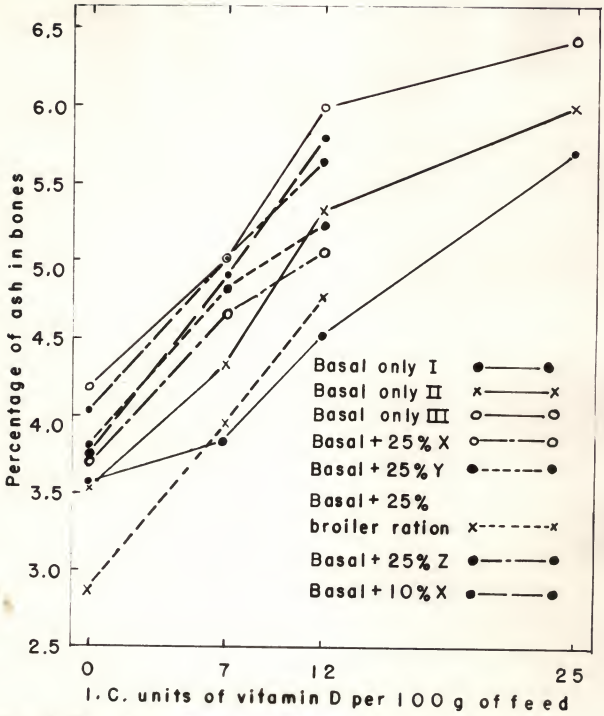

Fig. 3. Percentage of ash in the right toe of Trials I, II, and III.

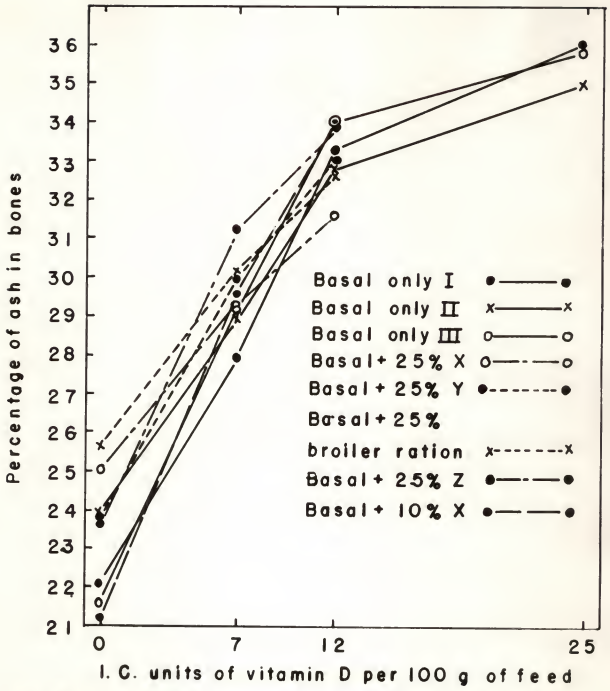

Fig. 4. Percentage of ash in the lower beak of Trials I, II, and III.

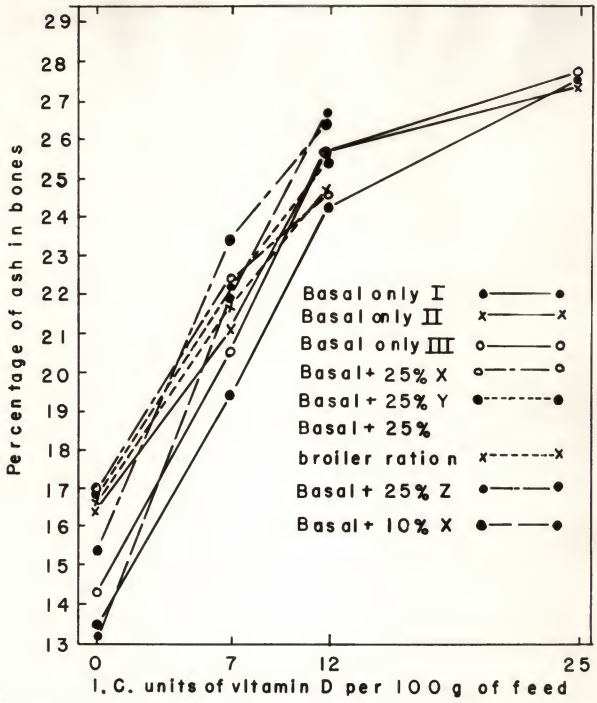

Fig. 5. Percentage of ash in the upper beak of Trials I, II, and III.

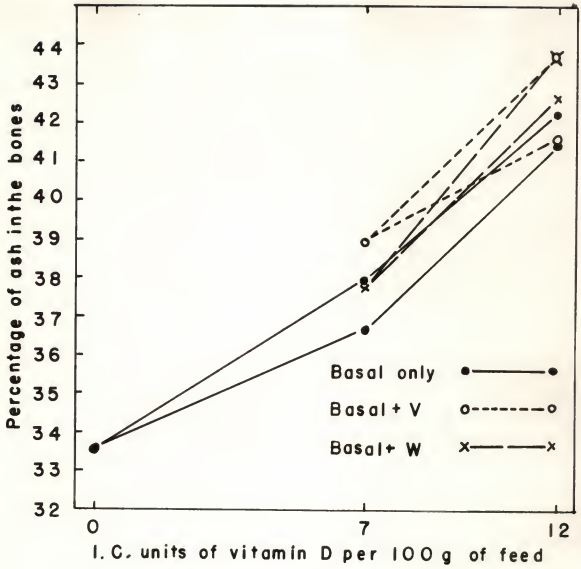

Fig. 6. Percentage of ash in the tibia of Trial IV.

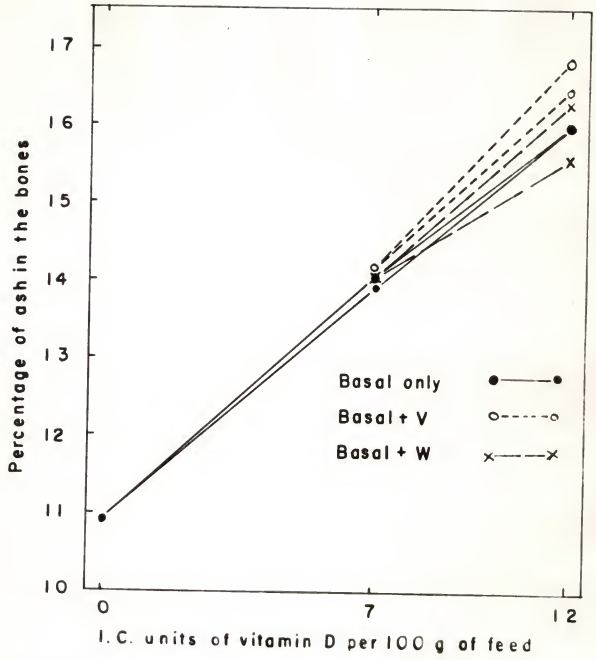

Fig. 7. Percentage of ash in the left toe of Trial IV.

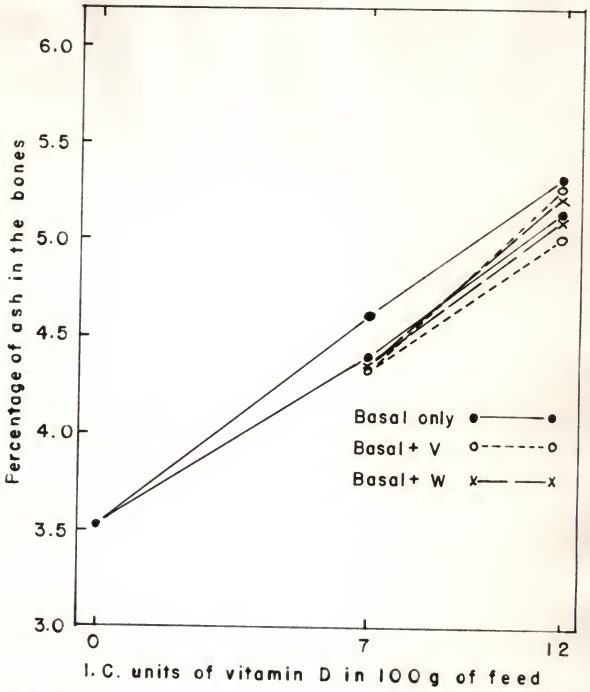

Fig. 8. Percentage of ash in the right toe of Trial IV.

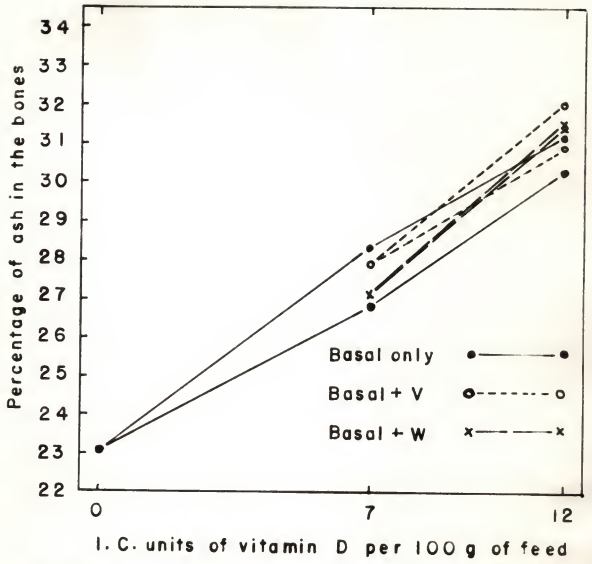

Fig. 9. Percentage of ash in the lower beak of Trial IV.

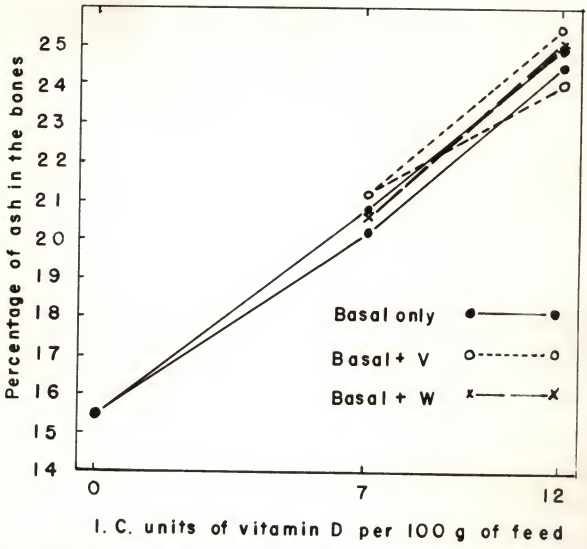

Fig. 10. Percentage of ash in the upper beak of Trial IV.

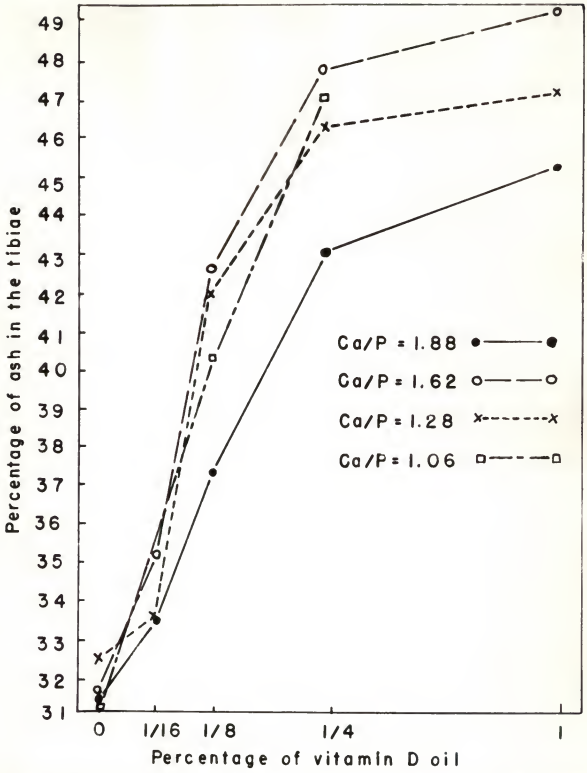

Fig.11. Graph of values reported by Griem et al showing the variation of bone ash due to variations in the Ca/P ratio of the basal rachitic ration (35).

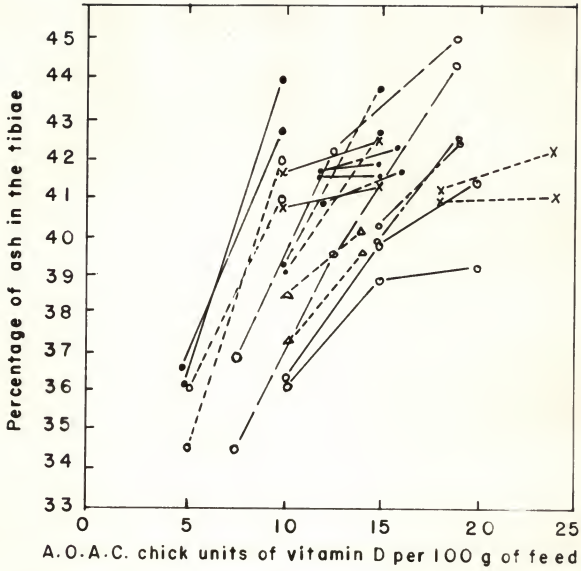

Fig. 12. Graph of results of the collaborative study of the chick bone ash method for the determination of vitamin D in 1940 (80). Lines having the same key are duplicate analyses.

DISCUSSION

In 1942 Fritz et al. (29) reported that:

> The assay of mixed feeds is difficult because of the relatively low potency of such samples. When a necessarily large sample is taken for assay, the effect of factors other than vitamin D may alter the calcification of the test groups. Influence upon calcification is not limited to minerals and vitamin D. When a sample constitutes as much as 25 per cent of the finished ration, it is questionable practice to compare results with reference groups receiving cod liver oil added to the A.O.A.C. basal diet. Extensive work with proper control groups is needed.

The usual objection to an assay such as the one proposed by Griem, the analysis of vitamin D in the prepared feed, is that the differences in the Ca/P ratio will cause variations in the bone ash which would be mistaken for vitamin D (11, 30, 34). Considerable work has been done on the effect of variations of the Ca/P ratio on the bone ash. Jones (50) and Griem et al. (35) have shown that there can be a wide variation in Ca/P ratio before it causes a significant difference in the bone ash (Fig. 11). In their experiments (using the former basal ration in which the Ca/P ratio was 1.28), Griem et al. found that the ratio could go up to 1.62 before significant differences were encountered. It was found, in Trial II of the present study, that the Ca/P ratio of the final mixture probably cannot exceed 1.25 without causing appreciable differences. Since the present basal ration has a Ca/P ratio of only 1.0, this is as much above the basal ratio as the 1.62 reported by Griem et al. It is doubtful that in an assay of a broiler or starter ration, with a maximum Ca/P ratio

of approximately 2, that the ratio of the final ration will ever be as high as 1.2, since the feed under assay will seldom constitute more than 15 per cent of the final ration.

One of the factors studied in the present investigation was the effect of the differences in Ca/P ratio on the assay of vitamin D in mixed feeds and, if necessary, find a method to adjust for the effect of these differences. When a feed with a Ca/P ratio of approximately 2.6, such as a laying mash, was added to the basal ration, it produced calcification greater than that caused by the basal ration alone. Therefore it was believed that if the proper amount of potassium phosphate were added to the feed in order to lower the Ca/P ratio to 1.0 (the same as that of the basal ration) this source of error might be eliminated. However, the results of Trial III indicate that this did not bring about the desired results, since the higher mineral content of the feed still caused a high ash content of the bones even though the Ca/P ratio was low.

If, instead of changing the Ca/P ratio of the feed to be assayed, calcium carbonate were added to the basal ration in order to raise the Ca/P ratio to that of the feed under assay, the accuracy of the determinations might be increased. However this increases the cost of the determinations, since a series of reference groups would have to be used for each feed assayed.

As stated by Fritz et al. (29) there probably are some factors that influence calcification, but these were not specifically considered in the present study.

An attempt was made in Trial IV, by the use of duplicate analyses, to determine how much of the difference in ash content of the bones could be normal variation and how much was due to differences in the feeds studied. It was found that only in a few cases did the duplicate analyses give the same results, and in some cases the differences were appreciable. When, however, the results of these duplicate determinations and those of the A.O.A.C. collaborative studies (3, 80, Fig. 12), were compared it was found that the proposed method was essentially as satisfactory as the official method. The average differences between the duplicate analyses of the collaborative studies (3, 80) were 1.134 and 0.975 per cent, respectively, whereas the average difference in the tibia ash content in the present study was 1.105 per cent. Thus it would seem that the method could be applied to the determination of vitamin D in chick starter and broiler mashes for control purposes, and possibly with some modifications, such as discussed previously, it might even be applied to layer mashes.

Before the proposed method for the determination of vitamin D in mixed feeds can be established, it is necessary to determine fully the accuracy of the method. A statistical study of the within group as compared to between group sources of variation is needed. This probably should consist of feeding four or more feeds at three or more levels. There should be at least three replications. Using twenty chicks in each group, this would require about forty pens and 800 chicks. A collaborative study of the proposed method by several laboratories also would be

advantageous, since only a few assays could be made in the present study.

The results of the present study are in agreement with those reported by Wei (82) in that the beak ash method seems to be a satisfactory method for the determination of vitamin D. This should be a logical criterion of calcification, since in many cases a soft rubbery beak is one of the first symptoms of rickets in chicks. This method seems to be nearly as satisfactory as the tibia ash method and better than the green and extracted toe ash methods, since the range between the minimum and maximum ash content of the beak is approximately the same as that found in the tibia and is much greater than that of the toe, either green or extracted. The beak ash method has the advantage that the beak is somewhat easier to remove and clean than is the tibia; however the beak is not as easy to remove as the toe. The beak ash method has the disadvantage that the total weight of the beaks is only about ten per cent of that of the tibia and, therefore, greater errors are likely to develop in the weighing of the bones and the ash.

SUMMARY

The purpose of the present study was to determine whether a modification of the A.O.A.C. method for the determination of vitamin D in poultry feed supplements could be applied to the assay of mixed feeds. The method used had been proposed by

Griem in 1935, but a library search disclosed no further work along that line.

In the proposed method the feed under assay was diluted with the A.O.A.C. basal rachitic ration and the calcification produced in chicks fed this ration was compared to the calcification produced in chicks fed the basal ration supplemented with the U.S.P. Reference Standard Vitamin D oil. The criteria of calcification used in this study were the ash contents of the tibia, the toe (both extracted and green), the upper beak, and the lower beak. In Trial I, the green toe also was ashed on the dry basis, but this was less satisfactory than the other criteria and was not used in the later trials.

Several different feeds were used to study the various factors that have an influence on calcification in the chick. Since the Ca/P ratio probably has the greatest influence on calcification, the ratio was varied from 1.02 to 2.57 in the feeds that were used. In general it was found that an increase in the Ca/P ratio caused an increase in the percentage of bone ash.

The precision and accuracy of the determinations using all of the criteria, except the green toe, were approximately the same. However, due to a smaller range between the minimum and maximum ash content of the green toe, the results obtained when using this criterion were not as satisfactory as the others. The variations between trials was also greater (Fig. 3). The findings of this study agree with those of Wei (82) that the ash content of either the upper or lower beak is a satisfactory criterion for the determination of vitamin D.

The results of this study indicate that this method for the determination of vitamin D in mixed feeds, in which the Ca/P ratio does not exceed 2.0, was as satisfactory as the official method for the determination of vitamin D in feed supplements.

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APPENDIX

Table 5. Calcium and phosphorus analysis of the rations.

Feed and trial	Calcium	Phosphorus	Ca/P ratio	Ca/P ratio in final ration
Broiler (I)	1.02	0.787	1.30	1.057
Basal (I)	0.864	0.885	0.976	0.976
Basal (II)	0.887	0.855	1.075	1.075
X (II)	1.14	0.575	1.98	1.301
Y (II)	1.475	0.575	2.57	1.449
X (III)	1.22	0.636	1.92	1.06
Z (III)	1.33	1.30	1.02	0.978
Basal (III)	0.82	0.85	0.964	0.964
Basal (IV)	0.795	0.848	0.936	0.936
V (IV)	1.02	0.787	1.30	
W (IV)	1.32	0.762	1.73	

Feed X has a Ca/P ratio somewhere between that of a starting mash and a laying mash while feed Y would correspond to a laying mash.

Table 10. Average weight gained by the chicks during the three-week feeding period.

Trial	Group	I.C. units of vitamin D per 100g of feed	Average Initial weight (grams)	Average final weight (grams)	Average weight gained (grams)
I	A	0	36.2	107.9	71.7
	B	25	39.2	148.1	108.8
	C	7	36.5	138.4	101.9
	D	12	38.2	158.3	120.1
	E	7	36.8	143.4	106.6
	F	12	38.6	165.7	127.1
	G	0	38.0	129.5	90.5
II	H	0	37.9	122.7	84.7
	I	7	38.9	143.0	104.1
	J	12	38.7	155.2	116.5
	K	25	39.5	168.5	129.0
	L	0	38.5	143.5	105.0
	M	7	38.4	167.7	129.3
	N	12	36.9	169.5	132.6
	O	0	38.8	160.0	121.2
	P	7	37.6	156.0	118.4
	Q	12	37.2	165.7	128.5
III	A'	0	34.4	96.5	62.1
	B'	7	35.3	137.5	102.2
	C'	12	35.0	135.3	100.3
	D'	25	34.7	135.4	100.7
	E'	0	36.0	109.0	73.0
	F'	7	37.1	136.0	98.9
	G'	12	35.7	153.8	118.1
	H'	0	36.5	105.0	68.5
	I'	7	34.4	137.7	103.3
	J'	12	36.7	141.4	103.7
IV	K'	0	40.8	101.9	61.1
	L'	7	40.8	136.5	95.7
	M'	7	40.3	111.1	70.8
	N'	12	38.6	134.3	95.7
	O'	12	39.9	134.7	94.8
	P'	7	41.3	143.5	102.2
	Q'	12	40.8	148.9	108.1
	R'	12	38.4	134.5	96.1
	S'	7	38.7	130.1	91.4
	T'	12	39.6	139.3	99.7
	U'	12	40.2	135.1	94.9

THE DETERMINATION OF VITAMIN D IN MIXED POULTRY FEEDS

by

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The purpose of the present study was to determine whether a modification of the Association of Official Agricultural Chemists method for the determination of vitamin D in poultry feed supplements could be applied to the assay of vitamin D in mixed feeds. The method was proposed in 1935 by Griem. He did some preliminary work, but a library search disclosed no further reports along that line.

In the proposed method, the feed under assay was diluted with the A.O.A.C. basal rachitic ration to make a final ration containing 7 - 12 I.C. units of vitamin per 100 g. and the calcification produced in bones of chicks fed this ration was compared to the calcification produced in bones of chicks fed the basal ration supplemented with the U.S.P. Reference Standard Vitamin D. The criteria of calcification used in this study were the ash contents of the fat- and moisture-free tibia, toe, upper beak, and lower beak and the green toe (unextracted and undried). In Trial I, the green toe also was ashed on the dry basis, but this was less satisfactory than the other criteria and was not used in the other trials.

Four trials were carried out in the present study. Since the Ca/P ratio of the feed is probably a factor that has an important influence on the calcification in the chick, the first three trials were set up largely to study the effect of varying the Ca/P ratio of the feed under assay. The ratio was varied from 1.02 to 2.57. It was found that in general an increase in

the Ca/P ratio caused an increase in the percentage of bone ash.

Due to the high vitamin D potency of most feeds (400 to 600 I.C. units per pound), it would seldom be necessary to add more than 15 per cent of the feed under assay to the basal ration in order to obtain the potency desired in the final ration. The Ca/P ratio of the final ration used in the assay probably would not exceed 1.20, since the Ca/P ratio of starter and broiler mashes is usually between 1.20 and 2.0. The results of the present study indicate that a ratio this high would not produce appreciable errors in the determination of vitamin D. However, when a feed, in which the Ca/P ratio was 2.57 (corresponding to a laying mash), was used, the errors were appreciable. It would then appear that the proposed method is satisfactory for the determination of vitamin D in starting and broiler feeds, while it might not be for laying feeds.

The precision and accuracy of the determinations using all of the criteria of calcification, except the green toe, were approximately the same. However, due to a smaller range between the minimum and maximum ash content of the green toe and a greater variation between the trials, the results obtained when using this criterion were not as satisfactory as the others.

The results of this study indicate that the ash content of either the upper or lower beak is a satisfactory criterion for the determination of vitamin D.